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A strategy for the generation of RNA competitors in competitive RT–PCR

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▼Several methods exist for the quantification of mRNAs. The classic methods, such as northern blots and RNAse protection assay, have been improved over the years and have provided reliable results but they share the drawback of having too low sensitivity for studies in small tissue samples. Recently the development of competitive RT–PCR assays has provided a valuable tool for this type of study (Ref. 1, 2), even if the reliability and accuracy of these assays has been a matter of intense discussion during the past years. Today the use of RNA competitors as internal standards in all steps of the reaction including the RT step is generally accepted as a reliable and sensitive method for the quantification of mRNAs (for review see Ref. 3). However, a major problem of competitive PCR in terms of effort and time is the generation of suitable competitor fragments. Here, we describe a streamlined method for the generation of RNA competitors by a straightforward PCR-based approach. The use of commercially avaible kits in most steps of the procedure allows the easy and efficient generation of RNA-competitors for use in competitive RT–PCR.

A schematic outline of the method is given in Figure 1. Three primer sites are used in this method, two nested forward primers (Fw1 and Fw2), with a distance of approximately 100 bp, and one reverse primer (Rev). The PCR assay using Fw1 and Rev should be optimized well (Ref. 4) and only generate a single product that is verified by sequencing or restriction analysis.

In the first round of PCR a fragment representing the native mRNA is generated by conventional RT–PCR using Fw1 and Rev as primers. This fragment is purified on a gel and eluted by use of an extraction kit [e.g. Quiaex II (Quiagen, Hilden, Germany)]. The purified first-round product is subjected to a second round of PCR using a hybrid primer (containing Fw1 at the 5′ end and Fw2 at the 3′ end) as forward primer and Rev as reverse primer. This second round of PCR results in a product containing Fw1- and Rev-primer sites that are about 100 bp closer to each other (i.e. the distance between Fw1 and Fw2), when compared with the native sequence.

Using a TA-cloning strategy [e.g. pGEM-T Vector system (Promega, Madison, Wisconsin, USA)], the fragment can be cloned easily into a plasmid vector containing suitable promotors for the *in vitro* transcription of RNA, like T7 or SP6. The identity of the insert and the extent of the deletion is finally verified by sequencing. After linearization of the plasmid, sense-competitor RNA is transcribed *in vitro* using a suitable kit [e.g. Riboprobe (Promega)] and the resulting RNA mimics are purified from the remaining plasmid template by DNAse 1 digestion. After dilution to appropriate concentration and control experiments that ensure equal and parallel amplification of competitor and native templates, the RNA competitor is ready for use in competitive RT–PCR (Ref. 2).

The method outlined above has been used in our laboratory to generate a series of RNA mimics for rat ATP receptors (Ref. 5). Figure 2 gives an example of the use of a competitor for rat $P2Y_2$ receptors generated with the method described here. Compared with other methods (Ref. 1, 2) the present strategy has several advantages. First, the use of a PCR-based, two-step strategy allows the use of existing and well-established PCR assays, which can be found in many laboratories. Second, no time-consuming and laborious cloning or subcloning steps are required except for the final cloning of the competitor. All steps of the procedure, including the cloning step, can be streamlined by convenient commercially avaible kits, thus, even making it possible for laboratories with little experience in molecular biology to set up competitive RT–PCR assays. In addition,

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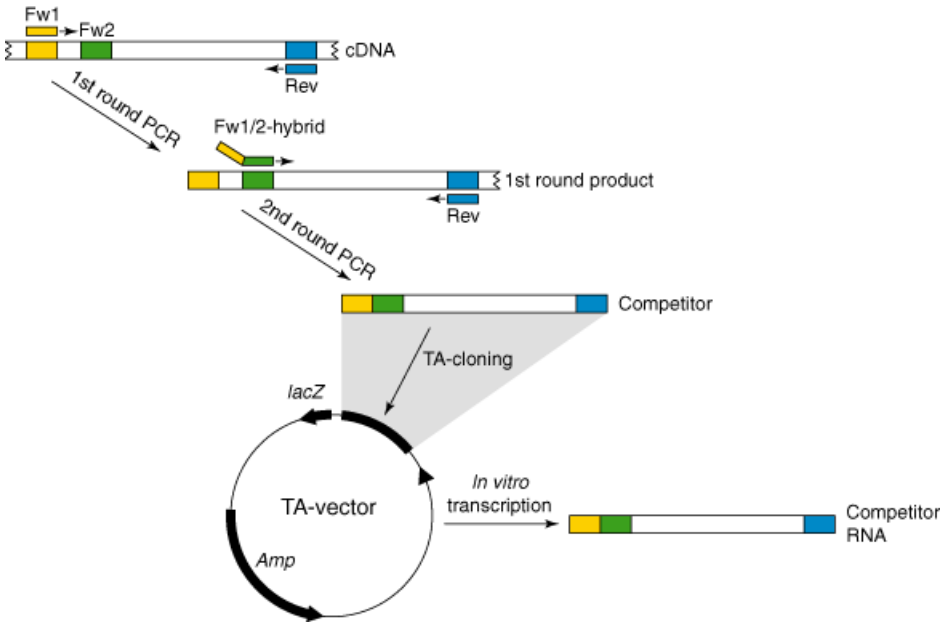

FIGURE 1. Flowchart for the generation of RNA-competitors. The primers used are: PCR-round 1. Fw1 as forward and Rev as reverse; PCR-round 2. Hybridprimer Fw1/Fw2 as forward and Rev as reverse. After TA-cloning the competitor fragment is transcribed *in vitro* into sense competitor RNA.

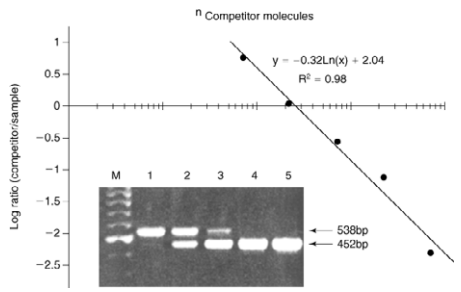

FIGURE 2. Example of a competitive RT–PCR method for rat $P2Y_2$ receptors. 200 ng of total RNA isolated from cultured rat smooth-muscle cells was mixed with a 1:10 dilution series of competitor RNA (ranging from 50 000 copies (lane 1) to 500 000$\times 10^3$ copies (lane 5). Competitive RT–PCR was performed as described

(Ref. 5). The PCR products were separated on a 2% agarose gel (insert) and analyzed densitometrically before the ratio of sample (538 bp) and competitor (452 bp) band intensities in each reaction was plotted against competitor numbers. Using linear regression, the eqivalence point (log ratio=0) in this experiment (i.e. the number of $P2Y_2$ mRNA molecules in the sample) was determined as 3160$\times 10^3$ copies/μg total RNA. M=100 bp size marker.

the use of competitors that yield a 100 bp deletion compared with the native sequence, combines convenient analysis on conventional agarose gels with minimal sequence differences to the native RNA, thus, ensuring parallel amplification. We hope that this method will help to overcome some technical problems in establishing competitive RT–PCR assays.

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Products Used

pGEM-T vector system: pGEM-T vector system from Promega Corporation

Riboprobe: Riboprobe from Promega Corporation